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Identification of the Neoplastically Transformed Cells in Marek's Disease Herpesvirus-Induced Lymphomas: Recognition by the Monoclonal Antibody AV37

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Understanding the interactions between herpesviruses and their host cells and also the interactions between neoplastically transformed cells and the host immune system is fundamental to understanding the mechanisms of herpesvirus oncology. However, this has been difficult as no animal models of herpesvirus-induced oncogenesis in the natural host exist in which neoplastically transformed cells are also definitively identified and may be studied in vivo. Marek's disease (MD) herpesvirus (MDV) of poultry, although a recognized natural oncogenic virus causing T-cell lymphomas, is no exception. In this work, we identify for the first time the neoplastically transformed cells in MD as the $CD4^+$ major histocompatibility complex (MHC) class I^{hi}, MHC class II^{hi}, interleukin-2 receptor α-chain-positive, $CD28^{lo/-}$, phosphoprotein 38-negative ($pp38^-$), glycoprotein B-negative (gB^-), αβ T-cell-receptor-positive (TCR^+) cells which uniquely overexpress a novel host-encoded extracellular antigen that is also expressed by MDV-transformed cell lines and recognized by the monoclonal antibody (MAb) AV37. Normal uninfected leukocytes and MD lymphoma cells were isolated directly ex vivo and examined by flow cytometry with MAb recognizing AV37, known leukocyte antigens, and MDV antigens pp38 and gB. CD28 mRNA was examined by PCR. Cell cycle distribution and in vitro survival were compared for each lymphoma cell population. We demonstrate for the first time that the antigen recognized by AV37 is expressed at very low levels by small minorities of uninfected leukocytes, whereas particular MD lymphoma cells uniquely express extremely high levels of the AV37 antigen; the $AV37^{hi}$ MD lymphoma cells fulfill the accepted criteria for neoplastic transformation in vivo (protection from cell death despite hyperproliferation, presence in all MD lymphomas, and not supportive of MDV production); the lymphoma environment is essential for $AV37^+$ MD lymphoma cell survival; pp38 is an antigen expressed during MDV-productive infection and is not expressed by neoplastically transformed cells in vivo; $AV37^+$ MD lymphoma cells have the putative immune evasion mechanism of CD28 down-regulation; $AV37^{hi}$ peripheral blood leukocytes appear early after MDV infection in both MD-resistant and -susceptible chickens; and analysis of TCR variable β chain gene family expression suggests that MD lymphomas have polyclonal origins. Identification of the neoplastically transformed cells in MD facilitates a detailed understanding of MD pathogenesis and also improves the utility of MD as a general model for herpesvirus oncology.

Herpesviruses establish lifelong latent infections, and many are associated with neoplasia, particularly lymphomas (e.g., Burkitt's lymphoma and anaplastic large cell lymphoma), in humans and other species (30). However, tumor formation after oncogenic herpesvirus infection is not obligatory; many individuals survive lifelong latent herpesvirus infections without clinical disease. Although poorly understood, host genotype and host immune function are essential in determining whether or not tumors occur after herpesvirus infection (6, 8, 12, 24, 44, 49, 58, 71, 96). However, herpesvirus tumorigenesis is multifactorial and understanding the cellular and molecular mechanisms fundamental to resistance or susceptibility has been difficult. Herpesvirus tumorigenesis results from complex interactions first between virus and host cells, then between infected cells and the immune system, and lastly between neoplastically transformed cells and the immune system.

Much work has addressed the first two interactions for a number of herpesviruses (82). However, the complex interactions between the immune system and neoplastically transformed cells are poorly understood for three main reasons. First, herpesvirus tumors are not clonal but are heterogeneous mixtures of both neoplastically transformed cells and immune cells attracted to the tumor, presumably as part of an inflammatory response. Identifying the neoplastically transformed cells within the tumor is essential to achieving a mechanistic understanding of herpesvirus tumorigenesis. Second, even when the neoplastically transformed cells may be identified, isolation from the tumor separates them from essential cellular and soluble signals. Studying human herpesvirus-induced neoplasia in vivo is difficult; human herpesvirus-transformed cells are generally studied in vitro, isolated from their natural environment. Third, no natural animal herpesvirus-induced tumor models have been reported for which the neoplastically transformed cells are definitively identified and can be studied within the tumor context. Artificial murine models exist, but these rely on immunodeficient strains of mice injected with clonal cell populations cultured in vitro. Such murine models may not reflect immunity to neoplastically transformed cells in a natural disease.

Marek's disease (MD) virus (MDV) is a naturally occurring

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oncogenic alpha-herpesvirus of chickens. MD has contributed greatly to the understanding of herpesvirus oncogenicity (30), and it is an important commercial poultry pathogen for which vaccinal control is cyclically problematic (106, 107). Typically, MD (i.e., lymphoma formation) depends on host genetics (12) and immune function (reviewed by Calnek in reference 18). Initial MDV infection is B lymphotrophic and cytolytic and is followed by lifelong latency in T lymphocytes. After infection with any given MDV strain, gross T-cell-predominant lymphomas form only in individual chickens that have susceptible genotypes (18). MDV is an accepted and naturally occurring in vivo animal model of herpesvirus oncology (18, 30, 105), but in common with other animal models, a major impediment to its utility is that the neoplastically transformed cells in MD lymphoma have not been identified.

Previous work has suggested that a monoclonal antibody (MAb), AV37, recognizing a novel surface antigen expressed by MDV-transformed cell lines (MDCC) in vitro, identifies the neoplastically transformed cells in MD lymphomas (14, 15, 85). However, $AV37^+$ cells are present not only within the developing lymphomas of genetically susceptible chickens but also in the organs of resistant genotypes of chickens up to 21 days post-MDV infection (14). This suggests that $AV37^+$ cells may not be neoplastically transformed; the antigen recognized might merely be expressed by lymphocytes that are part of the inflammatory response.

In this work our aim was to establish whether $AV37^+$ cells are the neoplastically transformed cells in MD lymphomas. Many definitions of neoplastic transformation are based on in vitro characteristics. However, the neoplastically transformed cells in MD lymphomas must conform to four previously established criteria for neoplastic transformation in vivo. First, their proliferation must be uncontrolled. Second, despite this they must be protected from cell death in situ (31). Third, because herpesviruses have both productive and latent life cycles and because the productive herpesvirus life cycle is lytic, they must not be productively infected (17, 82). Fourth, they must be present in all MD lymphomas. Finally, it would be greatly advantageous (but not critical to the definition of neoplastic transformation in vivo) if we could identify potential immune escape mechanisms of neoplastically transformed MD lymphoma cells. Such mechanisms would be essential in MD because MDV translates proteins in latency (62), and the neoplastically transformed cells must be latently MDV infected (17). Somehow, the neoplastically transformed cells must avoid inducing an immune response to such latent MDV antigens.

Here, for the first time, we identify the neoplastically transformed cells in MD lymphomas. The neoplastically transformed cells overexpress a host-encoded antigen recognized by the MAb AV37. We demonstrate that the lymphoma environment is essential for $AV37^+$ MD lymphoma cell survival. In doing so, we demonstrate that MDV phosphoprotein 38 (pp38), although suggested as a putative oncogene (26, 110), is a productive antigen and by definition cannot be expressed by neoplastically transformed cells in vivo. We also show that neoplastic transformation occurs early after MDV infection in both MD-resistant and -susceptible chickens. Based on T-cell receptor (TCR) variable β chain (TCRVB) gene family expression, we demonstrate for the first time that MD lymphomas are polyclonal. Identification of the neoplastically transformed cells in MD not only enables future studies of MD lymphoma pathogenesis but also enhances the utility of MD as an animal model for herpesvirus-induced lymphoma.

MATERIALS AND METHODS

Chickens and virus. Specific-pathogen-free and anti-MDV maternal antibody-free chickens were bred, raised, and housed in isolated accommodation at the Institute for Animal Health (IAH), Compton, United Kingdom. Both outbred (Rhode Island Red [RIR]) and inbred chickens were used. The inbred chicken lines have been selected over many generations for susceptibility (lines 7_2 and 15I) or resistance (lines 6_1 and N) to MD. Fourteen-day-old chickens were infected with HPRS-16 MDV exactly as described previously (14). All animal work was done under and complied with United Kingdom Home Office regulations.

Preparation of cell suspensions. Unless otherwise stated, all procedures were done at 4°C or on ice and all washes and suspensions were in phosphate-buffered saline with 0.4% bovine serum albumin fraction V (Sigma, Poole, United Kingdom) and 0.02% sodium azide (PBA). All chicken blood samples were obtained by jugular venipuncture with heparinized syringes. Blood from Holstein-Friesian cows (housed at the IAH), used as a source of negative-control peripheral blood leukocytes (PBL), was obtained by jugular venipuncture with heparinized syringes. Numbers of cells were estimated with a hemocytometer. In all cases, lymphomas were obtained from whichever nonlymphoid organ they occurred in, when individual line 7_2 chickens showed MD-specific clinical signs (from 40 days postinfection [dpi] onwards). The entire lymphoma mass was removed postmortem, placed immediately into PBA, and teased apart with forceps to obtain single-cell suspensions. PBL and lymphoma cells were purified by discontinuous density gradient centrifugation ($150 \times g$, 10 min, Ficoll-Paque [specific gravity, 1.083]; Pharmacia, Uppsala, Sweden). Cells were drawn from above the interface with a fine-tipped pipette, washed by centrifugation ($80 \times g$, 10 min) twice, and resuspended. Bone marrow (BM) was scraped from the femur, humerus, and sternum after the bones had been split with scissors, suspended, vortexed, and left to stand on ice (10 min). BM cells were removed from under the fat layer with a fine-tipped plastic pipette, pelleted by centrifugation ($150 \times g$, 10 min), and resuspended.

Cell lines. MDCC IAH8, HP9, HP18, HP89 (74), MSB-1 (39), and RPL-1 (66) were used and maintained as described previously (74). The chemically transformed fibroblast cell line CHCC-OU-2 was cultured and infected with HPRS-16 MDV as described previously (1).

MAbs. The MAbs used (Table 1) were either unconjugated or directly conjugated to phycoerythrin (PE), fluorescein-isothiocyanate (FITC) or 5(6) carboxyfluorescein-*N*-hydroxysuccinamide ester (FLUOS) (Boehringer Mannheim Biochemica, Mannheim, Germany). The secondary antibodies were conjugated to FITC, PE, magnetic beads, alkaline phosphatase, or horseradish peroxidase.

Flow cytometry. A FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) was used as described previously (15). Unless otherwise stated, all steps were carried out at 4°C and all dilutions and washes were in PBA. All immunostaining was done in 96-well U bottom plates (Nunc AS, Roskilde, Denmark) using 10^6 cells/well. After washing, the cells were pelleted by centrifugation ($80 \times g$, 2 min, 4°C). After centrifugation, a manual flick removed the wash buffer or MAb. For single-color immunostaining, the primary MAb (50 μl) was added to the appropriate well and the samples were incubated for 20 min. Cells were then washed twice, resuspended in goat anti-mouse immunoglobulin (Ig)-FITC (25 μl, 1:200), and incubated for 20 min. After immunostaining, cells were washed twice, resuspended in 100 μl of PBA, and added to 100 to 500 μl of FACSFlow in fluorescence-activated cell sorter tubes (Becton Dickinson) for analysis. Two-color immunostaining was as described above, except either mixtures of two primary MAbs of different isotypes, followed by mixtures of isotype-specific secondary fluorescent conjugates, or mixtures of two primary MAbs directly conjugated to either FITC or PE were used.

AV37 expression by uninfected cells. AV37 antigen expression by uninfected cells was examined in six separate experiments. DNA isolated from 10^6 cells from all samples was tested by PCR for MDV as described previously (15).

(i) Experiment 1. PBL were isolated from 10 21-day-old line 7_2 chickens and 10 cows. All PBL were immunostained with AV37; respiratory syncytial virus glycoprotein (RSVG)-MAb29, TRT6, or CZ3 (isotype-matched negative controls); or CC8 or CT4 (positive controls) as appropriate and examined by flow cytometry.

(ii) Experiment 2. PBL were isolated from four 14-day-old line 7_2 chickens and four cows, immunostained with AV37-FLUOS or IgG2a-FITC (negative control), and examined by flow cytometry.

TABLE 1. MAbs and antibody conjugates

MAb or polyclonal antibody (isotype or source)	Target antigen	Reference and/or source
MAbs		
2G11 (IgG1)	Chicken MHC class II β chain	47
AV7 (IgG1)	Chicken CD28	111
AV20 (IgG1)	Chicken B6 (Bu-1)	86
AV37 (IgG2a)	Novel molecule on MDCC HP9 and HP89	85
BD1 (IgG2a)	MDV pp38	54
CC8 (IgG1)	Bovine CD8	40
CC15 (IgG2a)	Bovine WC1	40
CT4 (IgG1)	Chicken CD4	20
CT8 (IgG1)	Chicken CD8 α chain	20
CVI-ChnL-68.1 (IgG1)	Chicken monocytes/macrophages	46
CZ3 (IgG2a)	Horse MHC class I	D. F. Antczak, personal communication
F21-2 (IgG1)	Chicken MHC class I α chain	89
F21-21 (IgG1)	Chicken MHC class I β_2 microglobulin	89
HB3 (IgG2b)	MDV gB	N. Ross, IAH, personal communication
IgG2a negative control (IgG2a)	Rat surface antigen on activated T helper cells	Serotech Ltd, Oxford, United Kingdom
LD16 (IgM)	Chicken IL-2R α	92 and P. Kaiser, personal communication
RSVG-MAb29 (IgG2a)	Bovine RSVG 90	G. Taylor, IAH, personal communication
RSVG-MAb30 (IgG1)	Bovine RSVG 90	G. Taylor, IAH, personal communication
TCR1 (IgG1)	Chicken TCRγδ	95
TCR2 (IgG1)	Chicken TCRαβ1	22
TCR3 (IgG1)	Chicken TCRαβ2	21
TRT6 (IgG2a)	Turkey rhinotracheitis virus glycoprotein	23
Polyclonal antibodies		
IgG (H+L)-PE-FITC (goat)	Mouse IgG (H+L)	Southern Biotechnology Associates, Inc.
IgG1-PE (goat)	Mouse IgG1	Southern Biotechnology Associates, Inc.
IgG2a-FITC (goat)	Mouse IgG2a	Southern Biotechnology Associates, Inc.
Goat anti-mouse IgG2b-PE (goat)	Mouse IgG2b	Southern Biotechnology Associates, Inc.
IgF (ab′)2-FITC (goat)	Mouse IgF (ab′)2	Southern Biotechnology Associates, Inc.
IgG2a + IgG2b-magnetic beads (rat)	Mouse IgG2a + IgG2b	Miltenyi Biotech, Bergich, Gladbach, Germany
IgG1-alkaline phosphatase (rabbit)	Mouse IgG1	DAKO Ltd.
IgG2a-horseradish peroxidase (rabbit)	Mouse IgG2a	DAKO Ltd.

(iii) Experiment 3. Spleen, thymus, and bursa were taken from four 14-day-old line 7_2 chickens. Cells, isolated by manual teasing of each organ followed by density gradient centrifugation, were immunostained with AV37-FLUOS or IgG2a-FITC (negative control) and examined by flow cytometry.

(iv) Experiment 4. BM cells were isolated from 21-day-old line 7_2 chickens, immunostained with AV37-FLUOS or IgG2a-FITC (negative control), and examined by flow cytometry.

(v) Experiment 5. PBL (10^8) were isolated from five line 7_2 and five RIR 42-day-old chickens, incubated with AV37 or RSVG-MAb29 (isotype-matched negative control), washed twice, incubated with anti-mouse IgG2a plus IgG2b conjugated to microbeads, and sorted by magnetic cell-sorting columns as described previously (15). PBL (10^5) from each sorted population were then incubated with Ig F(ab′)$_2$-FITC and analyzed by flow cytometry. Cytospin slides were prepared from the AV37$^+$ and AV37$^-$ cells, stained with May-Grunwald Giemsa stain (BDH, Merck Ltd., Poole, Leicstershire, United Kingdom), and examined by fluorescent and light microscopy.

(vi) Experiment 6. PBL (10^8) isolated from the chorioallantoic blood of three embryonic day 14 (E14) RIR embryos were magnetically sorted and then analyzed by flow cytometry (as described above).

Activation of isolated splenocytes and AV37 antigen expression. A sterile growth medium (Dulbecco's minimal essential medium containing 2.5% fetal calf serum, 0.1% penicillin-streptomycin, and 1% L-glutamine [Life Technologies Ltd., Paisley, United Kingdom]) was prepared, stored at 4°C, and used throughout. All incubations were at 41°C. Spleens were removed from one male and two female RIR chickens (70 days old); cells were isolated and suspended in growth medium (10^7 cells ml^{-1}) in two T80 flasks (total volume, 10 ml). To one flask of cells taken from each chicken, concanavalin A (ConA; Sigma) was added (final concentration, 20 μg ml^{-1}); the other was maintained without ConA. Three samples (2×10^6 cells) were taken from each flask and incubated separately in individual wells of a U bottom 96-well plate. To confirm ConA activation at 24 h of incubation, 10^5 cells were examined by flow cytometry for forward scatter (FSC) and side scatter (SSC), cells in the 96-well plates were pulsed with growth medium (10 μl) containing 1 μCi of tritiated thymidine, and ^{3}H incorporation was measured as described previously (5).

After 24 h, cell suspensions were removed from each flask and centrifuged and the medium was removed. The cells were washed twice with growth medium and resuspended (10^7 cells ml^{-1}) in fresh medium. The medium from the ConA-stimulated flasks was filtered by using Centriprep 100 (pore size, 0.2 μm) filters (Amicon; W. R. Grace & Co., Bedford, Mass.) according to the manufacturer's instructions, and the filtrate was retained as conditioned medium (CM). The ConA-stimulated cultures were subdivided into three equal samples in T25 flasks and incubated with 10, 15, or 20% of their own CM, respectively. Every 3 days, the cells in each flask were removed, centrifuged, and resuspended in fresh growth medium with CM at the appropriate concentration. After 1, 3, 7, 14, and 21 days of incubation, 2×10^5 cells were removed from each flask, incubated with either AV37 or RSVG-MAb29, washed twice and then incubated with goat anti-mouse Ig heavy plus light chains (H+L)-FITC. For each sample, corrected percentages of AV37$^+$ cells were calculated as described above.

Optimization of anti-MDV antigen MAbs for flow cytometry. BD1 and HB3 had not previously been used in flow cytometry. Therefore, they were titrated to optimal working dilutions with chick kidney cells (CKC) (2.5×10^6) cocultured (6 days, 37°C, 5% CO_2) with 10^5, 10^6, or 10^7 PBL isolated from latently MDV-infected line 7_2 chickens or with 10^5, 10^6, or 10^7 cells of MDCC HP9 and HP89. Because published reports are contradictory as to whether the MDV antigen pp38 (recognized by BD1) is expressed in latent and/or productive infection (26, 85, 110), CHCC-OU-2 cells were infected with HPRS-16 and examined by flow cytometry at both confluence (productive infection) and nonconfluence (latent infection) (1). Trypsin-EDTA (10 min, 37°C, 5% $C0_2$) was used to the produce single-cell suspensions of CKC and CHCC-OU-2.

Identification and DNA analysis of lymphoma cells. All washes and MAb dilutions were in PBA at 4°C; cells were analyzed by flow cytometry. To examine cytoplasmic antigen expression or DNA, cells were permeabilized with fluorescence-activated cell sorter permeabilizing solution (Becton Dickinson) by following the manufacturer's instructions.

TABLE 2. MD lymphomas examined and proportions of $AV37^{hi}$ $TCR2^{+}$ and $AV37^{hi}$ $TCR3^{+}$ cells

Bird no.	Lymphoma source	% $AV37^{hi}$ $TCR2^{+}$ cells	% $AV37^{hi}$ $TCR3^{+}$ cells
1	Nerve	13.4	7.7
2	Ovary	18.2	14.6
3	Nerve	10.6	4.5
4	Nerve	29.6	32.8
5	Heart	1.8	6.7
6	Jejunum	7.0	24.4
7	Nerve	7.4	3.6
8	Ovary	35.5	20.0
9	Nerve	23.8	5.9
10	Nerve	21.3	6.4
11	Ovary	7.3	18.2
12	Muscle	3.2	5.9
13	Periorbit	2.7	3.3
14	Liver	8.4	5.8
15	Heart	8.0	6.6
15	Nerve	14.9	7.6
15	Ovary	6.7	4.6
16	Ovary	3.1	29.3
16	Muscle	1.6	3.2
17	Muscle	27.1	14.4
17	Nerve	4.1	19.4

MD lymphoma cell composition and MDV antigen expression of MD lymphoma cells were investigated by using cells isolated from 14 lymphomas of line 7_2 chickens (Table 2, birds 1 to 14). These cells were incubated with MAbs 2G11, AV7, AV20, F21-21, TCR1, TCR2, TCR3, CT4, CT8, and LD16 recognizing leukocyte surface antigens or the negative-control MAb RSVG-MAb29 either in isolation or followed by either AV37 or the negative-control MAb RSVG-MAb30. The cells were then washed twice and incubated with goat anti-mouse IgG1 and/or goat anti-mouse IgG2a fluorescent conjugates. These cells were also examined for AV37 and either pp38 or glycoprotein B (gB) coexpression after being permeabilized, incubated with either BD1, HB3, or RSVG-MAb30 (isotype-matched negative control), washed, incubated with the appropriate PE conjugate, washed, incubated three times with normal mouse serum, washed, and finally incubated with AV37-FLUOS.

The 14 MD lymphomas (Table 2, birds 1 to 14) were also investigated for the presence of myeloid cells by processing frozen sections by immunohistochemistry as described previously (14). The MAbs used were CT4, CVI-ChnL-68.1, RSVG-MAb29, and RSVG-MAb30.

Leukocyte and MDV antigen expression were correlated with DNA ploidy by using a modification of the technique of Nicolletti et al. (67) on cells from the 14 lymphomas (Table 2, birds 1 to 14). Cells were incubated with either AV37, AV20, CT4, CT8, or TCR1 recognizing leukocyte antigens or the negative-control MAb CC8 or CC15 and directly conjugated to FITC or FLUOS before being permeabilized or permeabilized and then incubated with BD1, HB3, or the negative-control MAb RSVG-MAb29 followed by IgG (H+L)-FITC. The cells were then incubated with propidium iodide (25 $\mu g\ ml^{-1}$), washed twice, and examined by flow cytometry. Nonspecific isotype-matched MAbs, directly conjugated or unconjugated as appropriate, were used to quantify nonspecific immunostaining.

Leukocyte and MDV antigen coexpression were examined by incubating the cells from lymphomas (4 nerve, 3 ovary, 1 heart, 1 proventriculus, and 1 liver) taken from 10 line 7_2 chickens with either AV37, AV20, CT4, CT8, or TCR1 recognizing leukocyte antigens or the negative-control MAb CC8 or CC15 and directly conjugated to FITC or FLUOS. After incubation, the cells were washed, permeabilized, washed again, and incubated with BD1, HB3, or RSVG-MAb29. After washing again, the cells were incubated with the appropriate PE conjugates.

The issue of lymphoma clonality was further investigated by using cells isolated from three line 7_2 chickens which had multiple lymphomas (Table 2, birds 15 to 17). The isolated lymphoma cells were incubated with MAb AV37, TCR2, or TCR3 or the appropriate control MAb, washed, and incubated with the appropriate fluorescent conjugate before analysis by flow cytometry as described above.

DEX-induced PCD. Dexamethasone (DEX) was used to investigate the inherent resistance of MD lymphoma cell populations to programmed cell death (PCD) in vitro by using L-M Hahn medium with 10% fetal calf serum and 10% chick serum (LMH) and using LMH supplemented with 10^{-4} M DEX (Sigma) (LMH-DEX) (3). Cells isolated from three ovarian lymphomas were incubated in suspension (4×10^6 cells ml^{-1}) for 48 h under three different conditions: (i) LMH at 41°C with 5% CO_2, (ii) LMH-DEX at 41°C with 5% CO_2, and (iii) LMH at 4°C. At 0, 24, and 48 h, samples were analyzed by flow cytometry for cell phenotype and DNA ploidy. Samples of cells were also fixed in glutaraldehyde, processed, and examined by electron microscopy as described previously (5).

RNA preparation. All centrifugations were at $10{,}000 \times g$ (15 min, 4°C) unless otherwise stated. RNAzol B (AMS Biotechnology, Witney, United Kingdom) was used as described by the manufacturer's protocol to isolate RNA from 10^8 MDCC HP89, 10^8 chicken PBL, or 10^8 bovine PBL. RNA was resuspended in 1 mM $MgCl_2$ (20 μl) and treated with RNase-free DNase (2 U) (Boehringer Mannheim Biochemica) (37°C for 4 h and 95°C for 10 min). RNA was then precipitated with isopropanol (60 μl) with 3 M sodium acetate (2 μl) and stored at −70°C. When required, RNA was pelleted, washed (70% ethanol), vacuum dried, and resuspended in high-performance liquid chromatography-grade RNase-free water (100 μl).

CD28 transcription and expression by MDCC. Surface and cytoplasmic expression of CD28 was examined by flow cytometry in MDCC HP9, HP18, HP89, RPL-1, and MSB-1 with either MAb AV7 or the negative-control MAb RSVG-MAb29 and goat anti-mouse Ig-FITC. cDNA from MDCC HP9, HP18, and IAH8 and from ConA-stimulated T cells was provided by P. Kaiser (IAH). PCR primers were designed for exon sequences, spanning introns, of the chicken CD28 gene (111) (forward, GCC AGC CAA ACT GAC ATC TAC; reverse, TGA CTG CCT AGA AGC ACA CC), producing a 407-bp product from a cDNA template, and for exon sequences, spanning introns, of the chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (97) (forward, GGC CGT ATT GGC CGC C; reverse, CCC AGC CTT CTC CAT GG), producing a 652-bp PCR product from a genomic template and a 295-bp product from a cDNA template. Separate GAPDH and CD28 reverse transcription (RT)-PCRs were done on three separate occasions. RT and PCR amplification were done in a single reaction tube with a single thermostable DNA polymerase (recombinant *Tth* DNA polymerase and EZ buffer; Perkin Elmer) and a thermocycler (Hybaid Limited, Ashford, Middlesex, United Kingdom). The same PCR was used for the cDNA preparations. The PCR mixture contained RNA and/or cDNA (5 μl); each primer (20 pmol); 5 U of recombinant *Tth* DNA polymerase (2 μl); 10 mM (each) dATP, dCTP, dGTP, and dTTP (6 μl); 25 mM manganese acetate (5 μl); EZ buffer (10 μl); and high-performance liquid chromatography-grade nuclease-free water (final volume, 50 μl). The PCR cycle was 60°C for 30 min (RT); 94°C for 2 min; 40 cycles of 94°C for 1 min and 60°C for 1 min (amplification); and 60°C for 7 min. Products were run on a 1.0% agarose gel with 0.1% ethidium bromide (100 mV) and visualized with UV.

$AV37^{hi}$ cells in peripheral blood after MDV infection. Ten 14-day-old chickens from each of lines 6_1, 7_2, 15I, and N were infected with HPRS-16. Additional 14-day-old chickens (six from each line) were housed separately and mock infected as controls. Blood (0.5 ml) was taken at 3, 7, 14, 21, 28, 42, and 56 dpi. PBL were isolated, washed, incubated with either AV37 or the negative-control MAb RSVG-MAb29, incubated with goat anti-mouse Ig H+L-FITC, and analyzed by flow cytometry. The flow cytometer was compensated so that the maximum reading for the negative peak was at 10^1 relative fluorescence units; in total, 10^6 events were collected for each sample (based on the rare-event calculations described by Hoy [41]). For analysis, gates were set to collect only cells with a relative fluorescence of $>10^{1.5}$ (i.e., $AV37^{hi}$ as defined above). The corrected percentage of $AV37^{hi}$ PBL was then calculated for each chicken by subtracting the (negligible) percentage of RSVG-$MAb29^{+}$ cells from the percentage of $AV37^{+}$ cells. This percentage was compared between genotypes and between infected and uninfected chickens of the same genotype. To confirm the expected MDV infection status of each sample, DNA was isolated from 10^6 cells from each sample and tested by PCR as described previously (15).

Statistical analysis. Data were analyzed by one-way analysis of variance and Student's *t* test (as appropriate) with the Excel computer program (Microsoft Corporation, Seattle, Wash.).

RESULTS

Rare uninfected leukocytes, mitogen-activated leukocytes, fetal PBL, and hematopoietic cells express low levels of the AV37 antigen. Since the early 1970s, antigens expressed by MD lymphoma cells (Marek's associated tumor-specific antigens

[MATSA]) have been described by using polyclonal sera or MAbs (37, 43, 52, 55, 59, 63, 64, 68, 84, 91, 98, 100, 108). All differed in molecular weight and according to the genotype of the host (52, 61, 80, 98). Because of the range of antibodies in polyclonal sera and the number of antigens expressed by whole cells to which MAb could be raised, it is likely that many unrelated antigens have been identified. One MATSA identified by a MAb was suggested to be an activation antigen, i.e., it was up-regulated on splenocytes after mitogen activation (60). We investigated the possibility that the AV37 MAb recognized an antigen on a range of uninfected lymphocytes or was up-regulated by activated lymphocytes as well as by MD lymphoma cells. Because (by definition) oncofetal tumor antigens are expressed at high levels during ontogeny (25), we also examined BM cells and embryonic PBL as sources of lymphoid precursor and/or stem cells. To ensure that any identified $AV37^+$ cells were not due to unexpected MDV infection, all samples were confirmed to be MDV negative by PCR.

In six separate experiments (see Materials and Methods), uninfected PBL, BM cells, splenocytes, thymocytes, bursacytes, and E14 PBL were examined by flow cytometry with AV37. All cell types had a small subpopulation with low-level specific AV37 staining ($AV37^{lo}$) (a representative histogram is shown in Fig. 1A). This subpopulation was absent when nonspecific MAbs were used or when AV37 was used on bovine PBL; the positive-control MAbs CC8 and CT4 always gave distinct positive peaks when used on bovine and chicken cells, respectively. Both adult and E14 PBL could be enriched for $AV37^{lo}$ cells (a representative histogram is shown in Fig. 1B). The majority of the $AV37^{lo}$ cells were lymphoid and a few were thrombocytoid (Fig. 2A), but none were monocytoid.

Expression of the antigen recognized by AV37 was compared between unactivated lymphocytes and ConA-activated lymphocytes. After 24 h of ConA stimulation, ^3H uptake by unstimulated splenocytes was 2×10^3 cpm and ^3H uptake by ConA-stimulated splenocytes was 2×10^4 to 3×10^4 cpm. Also, more ConA-treated cells had SSC and FSC distributions typical of lymphoblasts (mean, 24.5% ± 1.8%) than the nontreated cells (mean, 11% ± 2.1%). The proportion of $AV37^{lo}$ cells increased after mitogen activation relative to time after activation (Fig. 1D) and to the concentration of CM (Fig. 1E), but it did not increase relative to the proportion of lymphoblastoid cells in the culture (Fig. 1F). Furthermore, the absolute expression level of the AV37 antigen did not increase statistically significantly, and even in the culture with the greatest response, the mean fluorescence intensity remained below 50 relative fluorescent units. The greatest increase in AV37 antigen expression was at 72 h in a 20% CM sample (6.13% increase in the percentage of $AV37^{lo}$ cells and 27% increase in the proportion of lymphoblastoid cells) (Fig. 1E and F).

Most MD lymphoma cells are $CD4^+$ (T helper) cells; a subpopulation of these cells uniquely overexpresses an antigen recognized by MAb AV37. Until now, the cellular composition of MD lymphomas has been inferred after analyzing lymphoid antigen expression by MDCC. Most MDCC are $CD4^+$, although $CD8^+$, $CD4^+$ $CD8^+$, and $TCR\gamma\delta^+$ MDCC exist (65). However, MDCC are clonal, free-floating suspensions of cells. MDCC represent only MD lymphoma cells that successfully made the in vitro transition. MDCC do not represent MD lymphomas as a whole; MD lymphomas are heterogeneous and structured (14). MD lymphomas examined directly by immunohistochemistry were shown to be clusters of $CD4^+$ (T helper) cells also recognized by the novel MAb AV37 and surrounded by $CD4^+$ $AV37^-$ cells and a few $CD8\alpha^+$ cells (14). The subpopulations of cells recognized by AV37 are suggested to be the neoplastically transformed cells within the MD lymphomas (14, 15, 85). However, we demonstrated that uninfected leukocytes could express the antigen recognized by AV37 (see above). To identify and quantify constituent cells, and also relative cell surface antigen expression, we examined MD lymphomas directly ex vivo by flow cytometry with a panel of MAbs recognizing leukocyte antigens, particularly MAb AV37 (see Materials and Methods).

A range of leukocyte phenotypes was present in MD lymphomas in relatively constant proportions. The majority of cells were $CD4^+$ $TCR2^+$ or $CD4^+$ $TCR3^+$. Although infrequent, $AV20^+$ (B cells), $CD8\alpha^+$, and $TCR1^+$ cells (potential cytotoxic lymphocytes) were present in all lymphomas (Fig. 3A). $TCR1^+$ cells were relatively frequent (4 and 10%, respectively) only in the lymphomas of the jejunum and the superficial pectoral muscle. The skin and gut are sites where $TCR\gamma\delta^+$ cells preferentially locate (13, 21, 48). $TCR1^+$ cells may have been present in greater frequencies in these lymphomas simply because of the location.

Cells recognized by AV37, regardless of antigen expression level ($AV37^+$), were common in all lymphomas we examined (mean, 43.7% ± 9.7%), and their phenotypic distribution reflected the phenotypic distribution of the lymphoma from which the cells were isolated (Fig 3B). Uniquely, we found MD lymphoma cells which express the AV37 antigen at high levels ($AV37^{hi}$) (a representative example is shown in Fig. 1C). However, $AV37^{hi}$ expression was restricted to a subset of the $CD4^+$ lymphoma cells; the $AV20^+$, $CD8\alpha^+$, and $TCR1^+$ lymphoma cells that expressed the AV37 antigen did so at a level similar ($AV37^{lo}$) to that of uninfected leukocytes (Fig. 4). The disparity between our previous findings that leukocytes in uninfected chickens are not recognized by AV37 with immunohistochemistry (14) and our data demonstrating $AV37^{lo}$ uninfected leukocytes with flow cytometry (Fig. 1) can be explained by the relatively poor sensitivity of immunohistochemistry in comparison to flow cytometry. Although neither $AV37^{hi}$ cell ubiquity nor AV37 antigen up-regulation are indicative of neoplastic transformation, both are consistent with the AV37 antigen being a tumor antigen.

Myeloid cells surround and are present within MD lymphoma cell foci. Although myeloid cells are associated with MD lymphomas (51, 69, 75, 78, 109) and may be significant in determining MD lymphoma progression or regression (7, 29, 34, 35, 38, 79, 93), they are not represented as MDCC. Because the density gradient separation we used to isolate MD lymphoma lymphoid cells was not optimal for myeloid cell isolation, we used immunohistochemistry (with MAb CVI-ChnL-68.1) to investigate myeloid cell presence and distribution in MD lymphoma.

Myeloid cells were found in all developing lymphoma foci examined by immunohistochemistry (a representative example is shown in Fig. 2B). Myeloid cells not only surrounded but also were present (to a lesser degree) within each individual lymphoma focus (Fig. 2C). The presence of myeloid cells suggests the possibility, even in MD-susceptible genotypes, of pri-

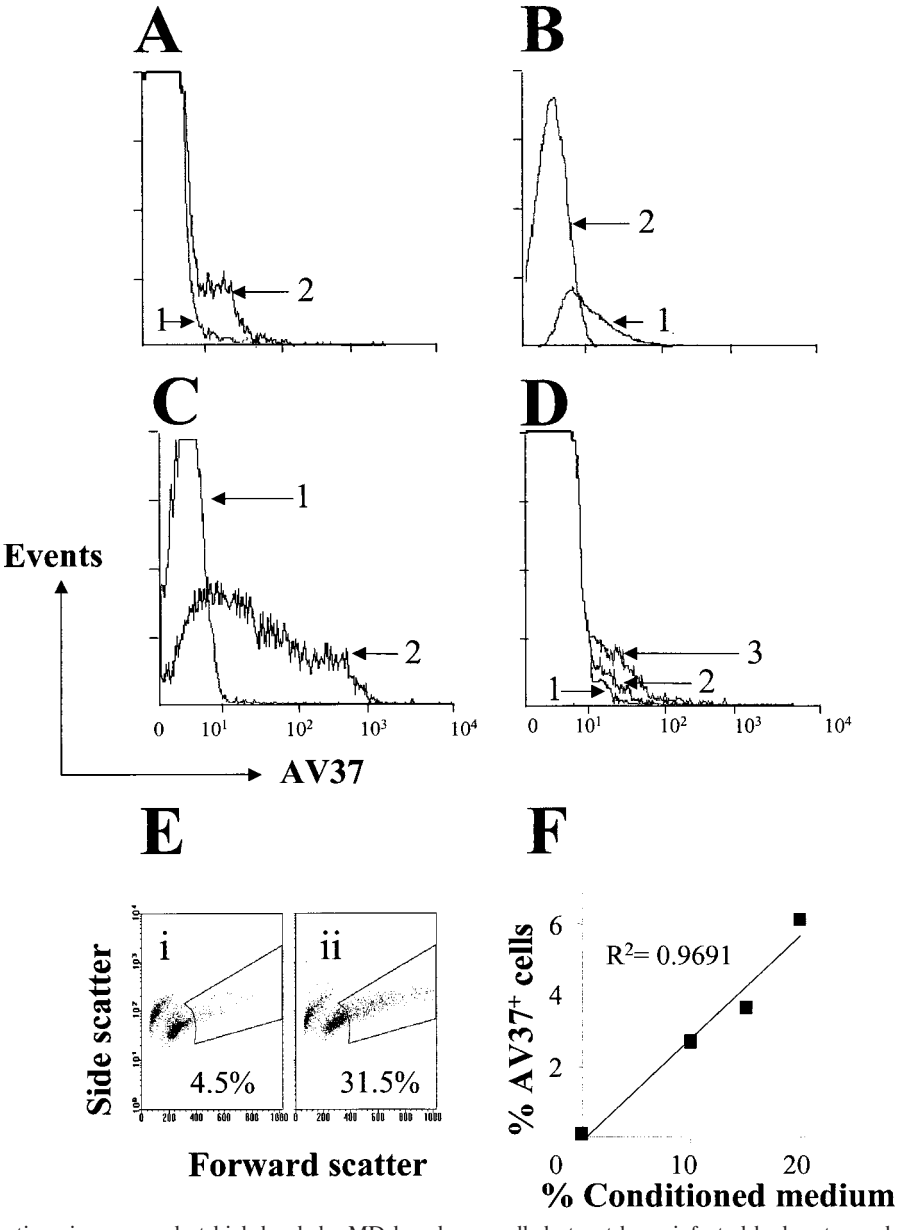
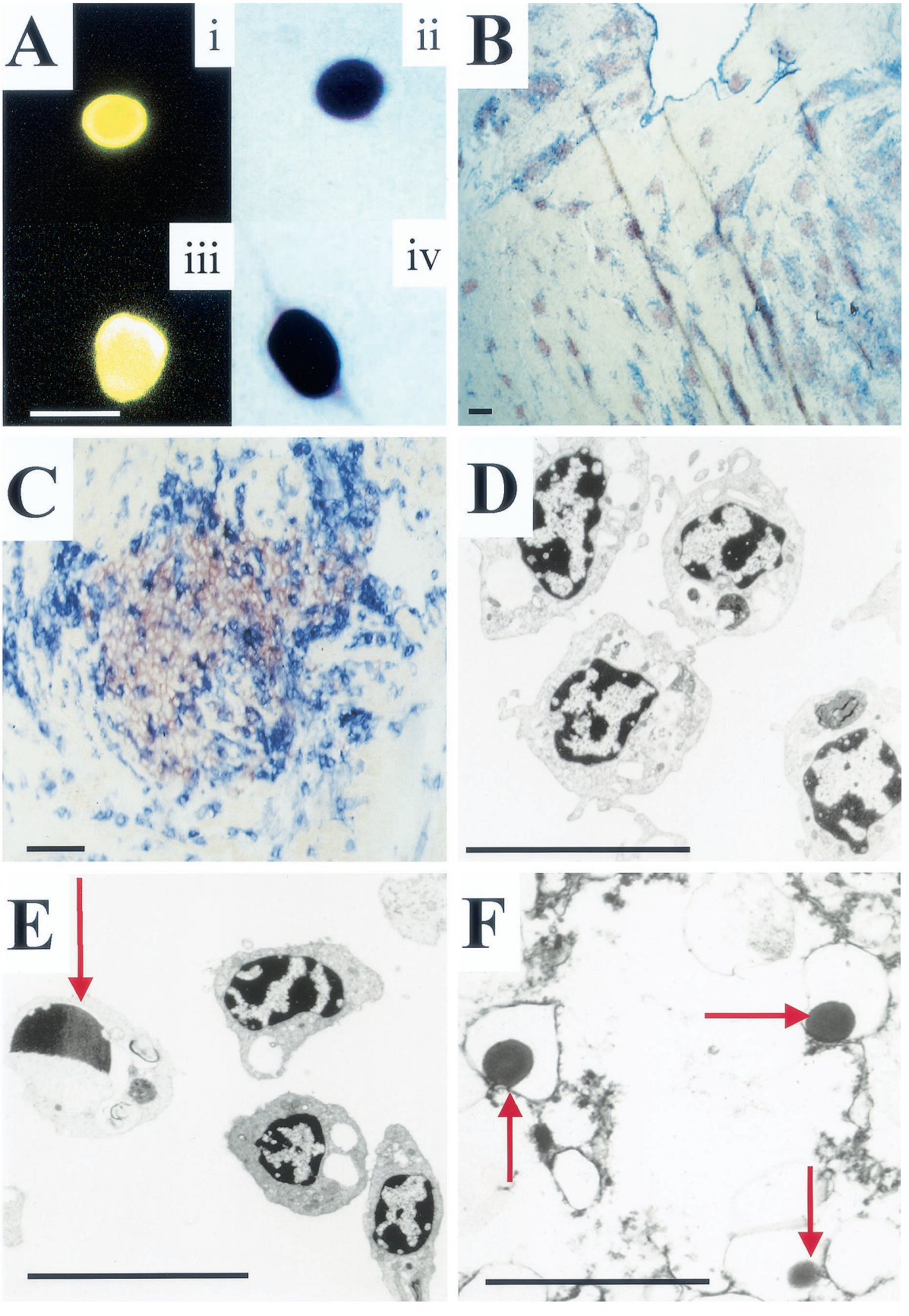


FIG. 1. The AV37 antigen is expressed at high levels by MD lymphoma cells but not by uninfected leukocytes or leukocytes after mitogen activation. (A) The MAb AV37 recognizes a host-encoded antigen expressed at low levels ($AV37^{lo}$) by a small proportion of uninfected PBL, splenocytes, thymocytes, bursacytes, BM cells, and E14 chorioallantoic blood leukocytes. The flow cytometry histogram overlay presented is of PBL stained with an isotype-matched control MAb (arrow 1) and AV37 (arrow 2). (B) $AV37^{lo}$ PBL (arrow 1) can be magnetically sorted from $AV37^{-}$ uninfected PBL (arrow 2). Results from magnetically sorted E14 chorioallantoic leukocytes are similar. (C) The antigen recognized by AV37 is grossly overexpressed ($AV37^{hi}$) by proportions of MD lymphoma cells. The flow cytometry histogram overlay presented is of a nerve lymphoma (Table 2, bird 1) immunostained with an isotype-matched control MAb (arrow 1) or AV37 (arrow 2). (D) Mitogen and CM activation of cultured uninfected splenocytes results only in small increases in both the proportion of cells recognized by AV37 and the level of AV37 antigen expression; the high level of AV37 antigen expression observed on lymphoma cells does not occur. All data on the figure are from male splenocytes, as described in Materials and Methods. Arrow 1, 0 h, 1.31% $AV37^{lo}$ cells; arrow 2, 24 h, 20% CM, 2.34% $AV37^{lo}$ cells; arrow 3, 72 h, 20% CM, 6.13% $AV37^{lo}$ cells. (E) The increases in $AV37^{lo}$ cell percentages in the cultures are not in proportion to the percentage increases in lymphoblastoid cells in the cultures. The percentage of lymphoblasts in the male splenocytes (as described above) after 24 h of culture with 20% CM; FSC versus SSC dot plots of untreated (i) and mitogen-treated (ii) cells. (F) The percentages of $AV37^{lo}$ cells in the cultures are proportional to the amount of CM used (72 h of culture, male splenocytes are shown as described above).

mary immune responses against neoplastically transformed cells given the appropriate cellular and soluble conditions.

$AV37^{hi}$ MD lymphoma cells are hyperproliferative and protected from cell death in vivo. Neoplastically transformed cells must disregulate the cell cycle and be hyperproliferative; however, they must be protected from the default pathway of activation-induced cell death (AICD) (31). The neoplastically transformed cells in MD are considered to be activated lymphocytes (18), so active mechanisms must protect such cells from AICD. We investigated cell death and proliferation di-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rectly ex vivo in different MD lymphoma cell phenotypes by measuring DNA ploidy. Cells with tetraploid DNA are in the S/G_2M (proliferative) phase of the cell cycle, most cells with diploid DNA are in the G_1 phase, and cells with subdiploid DNA are dying, usually by PCD (67).

$AV37^+$ lymphoma cells fit the two criteria for neoplastic transformation, i.e., hyperproliferation and protection from cell death (31). Relatively few (3.3% ± 0.6%) were subdiploid, despite a larger proportion being tetraploid (67.3% ± 7.0%) than diploid (30.1% ± 6.7%) (Fig. 3F). The $CD4^+$ cells (which included approximately 40% $AV37^+$ cells) had fewer subdiploid cells (2.0% ± 0.9%) than the $AV37^+$ cells but a smaller proportion of tetraploid (42.3% ± 4.5%) than diploid (55.8% ± 5.0%) cells. Many of the $AV20^+$ $CD8\alpha^+$, $TCR1^+$, $pp38^+$, and gB^+ cells were subdiploid (31.2% ± 8.3%, 16.3% ± 5.3%, 23.4% ± 6.1%, 37.3% ± 9.3%, and 37.3% ± 6.9%, respectively). Included in the $AV37^+$ group, but expressing the antigen only at low levels, were $AV20^+$, $CD8\alpha^+$, and $TCR1^+$ cells; many of these cells were subdiploid. The data were then analyzed for $AV37^{hi}$ cells only (defined as AV37 antigen expression greater than $10^{1.5}$ relative fluorescence units) (Fig. 1). The proportions of $AV37^{hi}$ cells that were tetraploid, diploid, and subdiploid were 72.7% ± 6.3%, 26.8% ± 5.9%, and 1.9% ± 0.5%, respectively.

The tetraploid-to-diploid-plus-subdiploid ratios (i.e., the proportions of proliferating cells relative to cells that are not replicating or are dying) for each cell population were as follows: $AV37^{hi}$, 2.5; $AV37^+$, 2.0; $CD4^+$, 0.7; $CD8\alpha^+$, 0.5; $TCR1^+$, 0.40; $AV20^+$, 0.7, $pp38^+$, 0.3; and gB^+, 0.4. The relative replication rates were 3.6 and 3 times greater in the $AV37^{hi}$ and $AV37^+$ cells, respectively, than in the (next highest) $CD4^+$ population as a whole. In short, of the lymphoma cell subpopulations we examined, the $AV37^{hi}$ cells had the lowest proportion of dying cells despite having by far the highest proportion of proliferating cells.

MDV antigens pp38 and gB are both expressed in productive MDV infection. Two MAbs, BD1 and HB3, recognizing MDV pp38 and gB, respectively, were used to investigate MDV antigen expression. BD1 and HB3 were titrated for use in flow cytometry with MDV-infected PBL, CKC, and the CHCC-OU-2 cell line. MDV-productive infection occurs only in confluent CHCC-OU-2 cells, whereas subconfluent CHCC-OU-2 cells support only latent MDV infection (1).

$BD1^+$ and $HB3^+$ cells could not be detected in MDV-infected subconfluent CHCC-OU-2 cell cultures, neoplastically transformed MDCC HP9 and HP89 cells (latently infected by definition), and PBL taken from MDV-infected chickens in clinical latency. In contrast, $BD1^+$ and $HB3^+$ cells could be detected in MDV-infected CKC, MDV-infected confluent CHCC-OU-2 cells (Fig. 5), and CKC which had been cocultured with MDCC HP9, HP89, or PBL taken from MDV-infected chickens in clinical latency.

It is generally accepted that gB is a late antigen expressed in productive MDV infection. However, the role of pp38 is controversial. It has been suggested that it is involved in neoplastic transformation (26, 110) and, conversely, that it is an early gene of cytolytic infection (85). Our work indicates that pp38 is associated with productive MDV infection rather than with latent infection. Our finding that the greatest proportions of both $pp38^+$ and gB^+ MD lymphoma cells are dying (Fig. 3) supports this conclusion.

Most B, $CD8\alpha^+$, and $TCR1^+$ cells, but few $CD4^+$ and no $AV37^{hi}$ MD lymphoma cells, are $pp38^+$ or gB^+. An essential criterion for neoplastically transformed cells in herpesvirus-induced neoplasia is that they are not productively infected because productive herpesvirus infection is cytolytic (17, 82). We identified productively infected cells in MD lymphomas as $pp38^+$ or gB^+.

In MD lymphomas, $pp38^+$ and gB^+ lymphoma cells are infrequent (3.73% ± 1.71% and 2.23% ± 1.20%, respectively) (Fig. 3C). No $AV37^{hi}$ cells, and only 2.5% of $CD4^+$ cells, were $pp38^+$ or gB^+ (Fig. 3D and E). $CD4^+$ and $AV37^+$ cells (equivalent to $AV37^{hi}$ cells in our work as discussed above) in MD lymphomas are positive for the MDV genome (15, 85). Lack of pp38 or gB expression suggests that this must be a latent infection. Furthermore, because absolutely no $AV37^{hi}$ cells were $pp38^+$ or gB^+, these cells are excellent candidates for the neoplastically transformed cells in MD.

Many $AV20^+$, $CD8\alpha^+$, and $TCR1^+$ cells were $pp38^+$ and/or gB^+ (Fig. 3E and F), and many are dying (subdiploid) (Fig. 3F). This death may be a direct consequence of productive infection, and the targeting of these cells by MDV may be an active immunosuppressive mechanism. Another possibility is that death of such cells may be due to the immune recognition of pp38 and gB (as described in reference 70). Regardless, the consequence is the same: $AV20^+$, $CD8\alpha^+$, and $TCR1^+$ cells would not be available within the MD lymphoma to kill neoplastically transformed cells.

$AV37^+$ lymphoma cells were not protected from death in vitro. Neoplastically transformed cells from some tumors are inherently immortal; they proliferate directly upon in vitro culture to form cell lines and are resistant to chemically induced PCD. In contrast, cells isolated from other tumors do not easily make the in vitro transition. Such cells are thought to

FIG. 2. (A) Photomicrographs of uninfected PBL and E14 leukocytes recognized by the MAb AV37 which are lymphocytoid (i and ii) or thrombocytoid (iii and iv). Figures shown are of dark-field (i and iii) and light-field (ii and iv) microscopy of uninfected adult PBL after magnetic sorting with AV37. Magnification, ×920. Bar = 15 μm. (B) Immunohistochemistry of the heart from an MDV-infected chicken (Table 2, bird 5). MD lymphomas form multiple foci, each focus is composed of mainly $CD4^+$ T cells (red) associated with fewer CVI-ChnL-68.1$^+$ (myeloid) cells (blue). Magnification, ×23. Bar = 100 μm. The myeloid cells mainly surround, although a few are present within, each developing MD lymphoma focus. (C) Single representative focus from the heart section (shown in panel B). $CD4^+$ cells are red, and CVI-ChnL-68.1$^+$ cells are blue. Magnification, ×368. Bar = 100 μm. (D to F) Electron microscopy of classical changes of PCD after ovarian MD lymphoma cells were incubated with 10^{-4} M DEX. Magnification, ×11,960. Bars = 15 μm. (D) Normal lymphoma cells are shown after 0 h of incubation. (E) After 34 h of incubation, there is a loss of cytoplasmic processes, rounding, increased cytoplasmic density, and nuclear condensation (arrow) typical of the early stages of PCD. (F) After 48 h of incubation, apoptotic bodies with condensed nuclear remnants (arrows) typical of the late stages of PCD are present.

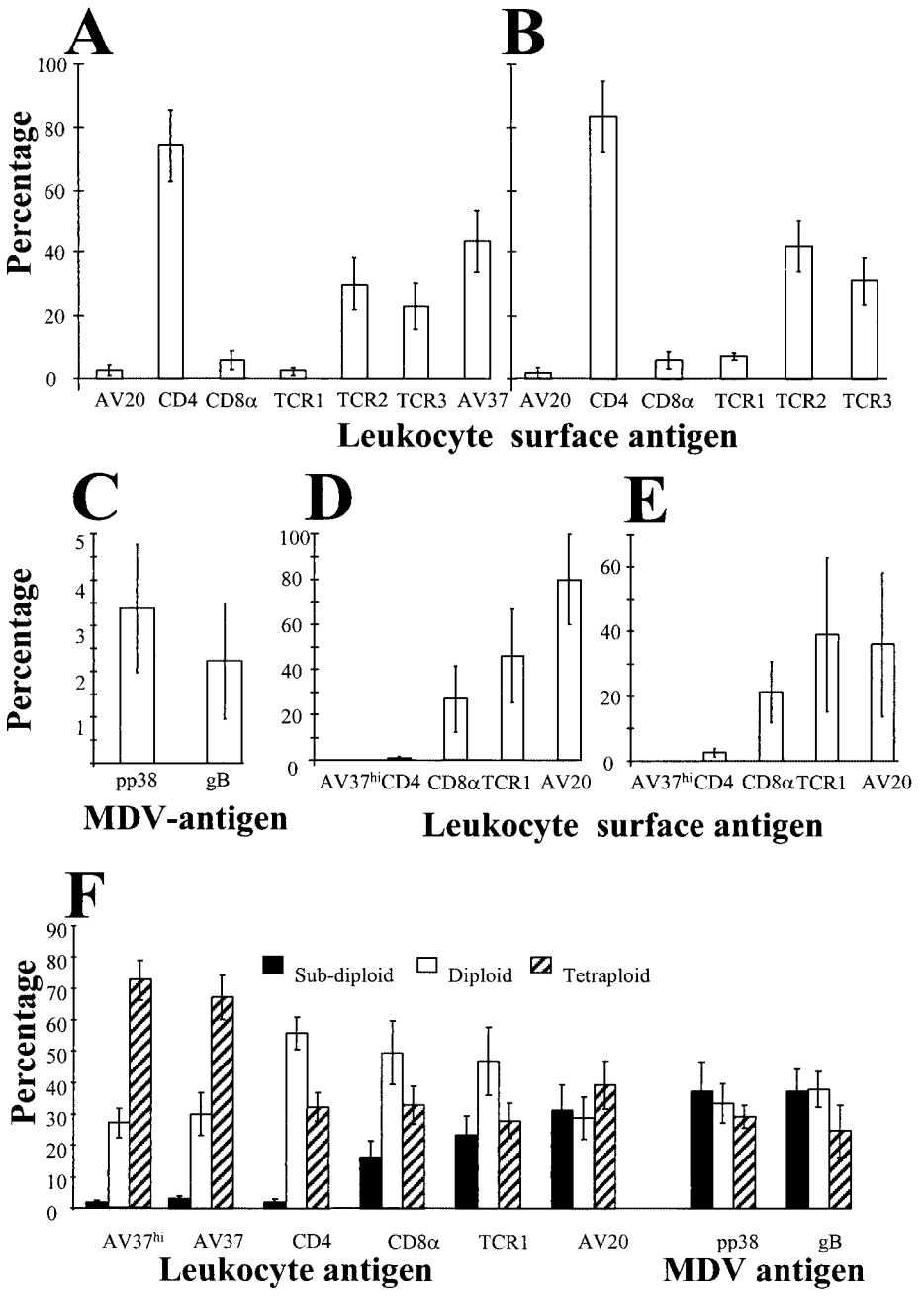

FIG. 3. Histograms of flow cytometry data of antigen expression by cell populations in MD lymphomas (Table 2, birds 1 to 14) (means ± standard errors of the mean). (A) The majority of MD lymphoma cells are $CD4^+$ and either $TCR2^+$ or $TCR3^+$; few are $AV20^+$, $CD8\alpha^+$, or $TCR1^+$. (B) The lymphoma cells recognized by AV37, regardless of expression level of the antigen ($AV37^+$), have phenotype distributions similar to those of lymphoma cells as a whole. (C) Few lymphoma cells are (MDV antigen) $pp38^+$ or gB^+. Large proportions of $AV20^+$, $CD8\alpha^+$, or $TCR1^+$ lymphoma cells, but few $CD4^+$ lymphoma cells and no cells expressing high levels of the AV37 antigen ($AV37^{hi}$), are $pp38^+$ (D) or gB^+ (E). (F) DNA ploidy of MD lymphoma cell populations (mean ± standard error of the mean). Few $AV37^{hi}$ and $AV37^+$ cells are subdiploid, relatively few are diploid, and most were tetraploid. Few $CD4^+$ cells are subdiploid, and most are diploid. Many $AV20^+$, $CD8\alpha^+$, and $TCR1^+$ cells are subdiploid, and most $pp38^+$ and gB^+ cells are subdiploid.

require additional transforming mutations and require media with specific growth factors, i.e., the local tumor environment is essential for their survival. We investigated whether the MD lymphoma environment was essential for the survival of cells in constituent subpopulations, any cell subpopulation was differentially dependent on the MD lymphoma environment, and any cell subpopulation was differentially resistant to chemically induced PCD in vitro. MD lymphoma cells were taken from three lymphomas ex vivo and incubated with CM with or without DEX. Dying cells were identified by electron microscopy. The proportions of dying cells in each lymphoma cell subpopulation were measured by flow cytometry (as described above).

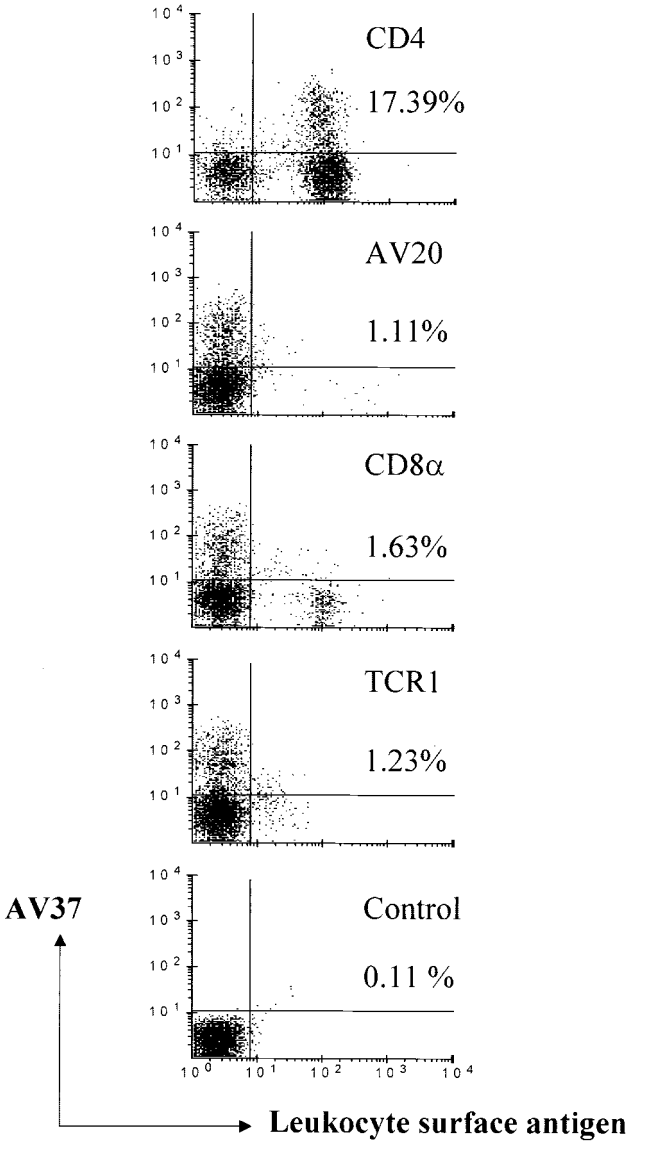

FIG. 4. Flow cytometry dot plots of AV37 antigen expression relative to leukocyte surface antigen expression by ovarian MD lymphoma cells (Table 2, bird 2). High-level AV37 antigen expression is exhibited by $CD4^+$ MD lymphoma cells but not by $AV20^+$, $CD8\alpha^+$, or $TCR1^+$ MD lymphoma cells. Leukocyte antigens are indicated in the top right of each dot plot. The percentages shown are percentages of cells coexpressing the leukocyte antigen and the AV37 antigen.

The results were similar for each lymphoma. When lymphoma cells were initially disaggregated and cultured, the $CD4^+$ cells had the lowest (mean, 5.5%) proportion of subdiploid cells and $AV37^+$ cells the second lowest (mean, 6.6%) proportion of subdiploid cells. Incubation at 4°C maintained the proportions of subdiploid cells in all phenotypes (data not shown). Incubation at 41°C increased the proportions of subdiploid cells in all phenotypes. After 48 h, 52% of $CD4^+$ cells and 60% of $AV37^+$ cells were subdiploid. $CD8\alpha^+$, $TCR1^+$, and $AV20^+$ cells could not be observed at 48 h (Fig. 6A). DEX (10^{-4} M) induced PCD in all MD lymphoma cell subpopulations, as demonstrated by an increase in apoptotic bodies (Fig. 2E and F) and by increased proportions of subdiploid $CD4^+$ (66%) and $AV37^+$ (80%) cells, respectively, after 48 h of incubation.

Our results indicate that $AV37^+$ MD lymphoma cells are not inherently immortal; like other lymphoma cell phenotypes, they die when cultured in vitro. To maintain their low death rates in the face of high proliferation in vivo, $AV37^+$ cells must rely on factors in the lymphoma environment that prohibit default AICD. The greater death rate we observed in the $AV37^+$ MD lymphoma cells than in the $CD4^+$ MD lymphoma cells is an expected consequence of a greater proliferation rate (i.e., AICD). The vital factors in the MD lymphoma could include soluble (e.g., cytokines) or cell surface molecules.

$AV37^+$ MD lymphoma cells down-regulate CD28 as a potential immune evasion mechanism. $AV37^+$ lymphoma cells fulfill four essential criteria of neoplastic transformation in vivo: hyperproliferation, protection from cell death, absence of productive MDV infection, and presence in all MD lymphomas examined. However, an additional criterion is that neoplastically transformed cells escape the immune system. This may be achieved by a number of means. Two of the most direct mechanisms are by avoiding antigen presentation and by avoiding costimulation; both are essential to the immune response. MD lymphoma cells are activated lymphocytes (18), and all $AV37^{hi}$ MD lymphoma cells are $CD4^+$ T helper cells (see above). Activated chicken $CD4^+$ lymphocytes up-regulate major histocompatibility complex (MHC) class I and class II, which are essential for antigen presentation to T killer cells and T helper cells, respectively; the essential immune costimulatory molecule CD28; and the interleukin-2 (IL-2) receptor α-chain (IL-2Rα) (unpublished data). IL-2R signaling, after ligation to IL-2, promotes naive T-cell proliferation, viability, and MHC class I up-regulation. In contrast, IL-2 binding to activated T cells that are being restimulated by antigen via their TCR promotes AICD and is a proposed mechanism for controlling immune responses (104).

We examined MHC class I and II, CD28, and IL-2Rα expression by flow cytometry. All data were normalized to account for differences in cell size and density. MHC class I and II and IL-2Rα expression were greater on $AV37^+$ than on $AV37^-$ MD lymphoma cells. CD28 was absent or expressed at lower levels on $AV37^+$ than on $AV37^-$ MD lymphoma cells (Fig. 6B and C). MHC class I and II and IL-2Rα expression were slightly greater, and CD28 expression was slightly lower, on $AV37^{hi}$ (defined above) lymphoma cells but not statistically significantly (data not shown). MDCC HP9, HP18, HP89, RPL-1, and MSB-1 cells were negative for cell membrane and cytoplasmic CD28. CD28 was expressed, but only at low levels, by MDCC IAH8 (data not shown).

The absence of cytoplasmic CD28 suggests down-regulation of CD28 mRNA. We investigated mRNA expression with MDCC because they are clonal populations of $AV37^{hi}$ cells. Unlike MD lymphomas, MDCC could be analyzed for CD28 mRNA without contamination from $AV37^{-/lo}$ cells (15). GAPDH mRNA was present in all samples. In contrast, CD28 mRNA was absent in MDCC HP9, HP18, and HP89 and bovine PBL but present in MDCC IAH8 (at a low level) and ConA-stimulated T cells (Fig. 6D), suggesting CD28 regulation at the mRNA level.

Thus, $AV37^+$ MD lymphoma cells have inherently high antigen-presenting potential to activated T killer and T helper

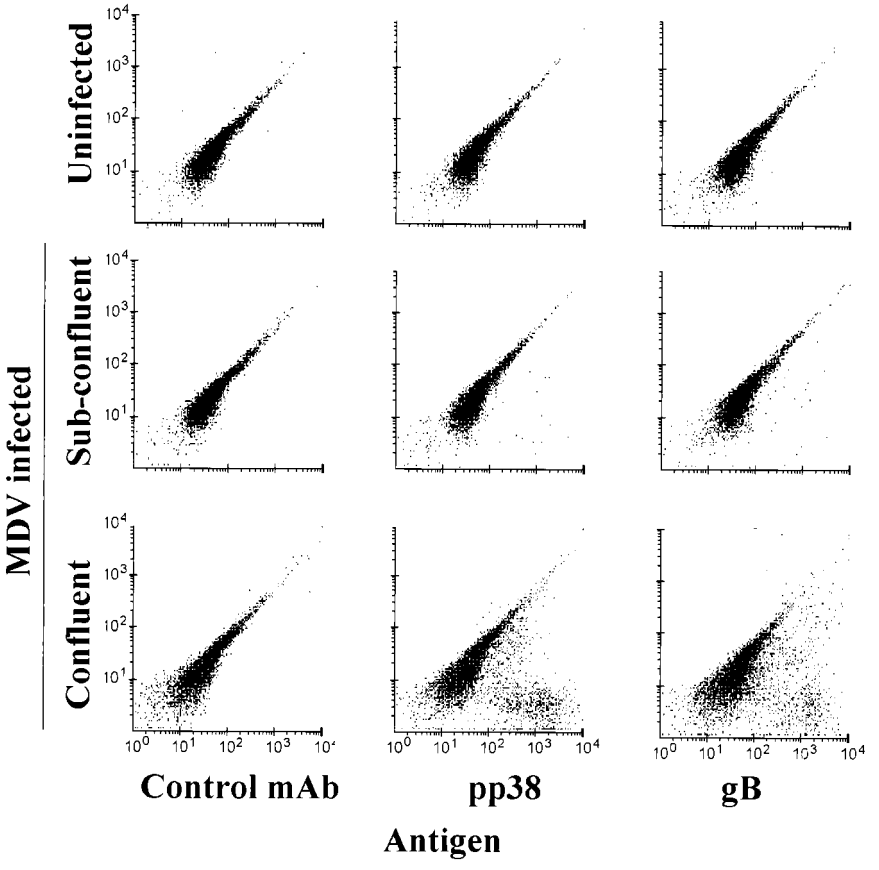

FIG. 5. Confluent but not subconfluent CHCC-OU2 cell cultures are supportive of MDV production after MDV infection. On the flow cytometry dot plots, the *x* axes show antigen expression and the *y* axes show MDV infection status and cell culture conditions. Uninfected confluent and subconfluent CHCC-OU2 cultures were identical and are represented by the same dot plots.

cells. They also have high IL-2 binding potential and consequently would be expected to be either highly proliferative (as demonstrated above) or, conversely, have a strong drive towards AICD. If promotion of AICD occurs, it appears to be overcome. A low level or absence of CD28 expression is one potential mechanism promoting $AV37^+$ lymphoma cell survival.

There is an $AV37^{hi}$ leukocytosis after MDV infection in both MD-resistant and -susceptible chickens. $AV37^{hi}$ MD lymphoma cells fulfill the criteria for neoplastically transformed cells in MD. However, $AV37^+$ cells have been demonstrated, with immunohistochemistry, after MDV infection in both MD-susceptible and -resistant chickens. As discussed above, these cells must have expressed high levels of the AV37 antigen to be detected by immunohistochemistry. However, after 21 dpi, the numbers of $AV37^+$ cells were markedly decreased in the resistant chickens; the few remaining $AV37^+$ cells were apoptotic (14). Together, these results suggest that neoplastic transformation after MDV infection is independent of susceptibility to MD and that lymphomas do not form in resistant chicken genotypes because such neoplastically transformed cells die. We investigated four lines of inbred chickens (6_1 and N [MD resistant] and 7_2 and 15I [MD susceptible]) after MDV infection for numbers of $AV37^{hi}$ PBL. Because we expected $AV37^{hi}$ PBL to be rare, rare-event flow cytometry analysis was used (41). PCR confirmed the expected MDV infection status in all chickens.

We detected no $AV37^{hi}$ PBL in uninfected chickens of any line at any sampling time. $AV37^{hi}$ PBL were detected, and were most frequent, in all chicken lines during the clinical cytolytic phase at 3 and 7 dpi. Although the proportion decreased, $AV37^{hi}$ PBL were still present after the disease entered clinical latency in line 7_2. Line 15I was similar to line 7_2, except that $AV37^{hi}$ PBL percentages were not present at 56 dpi. In the resistant lines, $AV37^{hi}$ PBL were absent at 21 dpi but present at 28 and 42 dpi. At 56 dpi, $AV37^{hi}$ PBL were present in line 6_1 but absent from line N (Fig. 7).

Our results suggest that neoplastic transformation begins early in MDV pathogenesis and occurs regardless of genetic susceptibility to lymphoma. Although it is theoretically possible that the $AV37^{hi}$ leukocytosis we measured is not caused by neoplasia in resistant chickens, our work described above and MDCC production from induced local lesions in MD-resistant chickens as early as 6 dpi (19) support our suggestion. Furthermore, the rank order for percentages of $AV37^{hi}$ PBL between the lines ($7_2 > 15I > 6_1 > N$) is identical to the rank order for susceptibility to lymphoid infiltration into nonlymphoid organs after MDV infection (14) and compatible with published data on susceptibility to MD (reviewed in references 4, 16, 57, and 76). The pattern of $AV37^{hi}$ leukocytosis is bi-

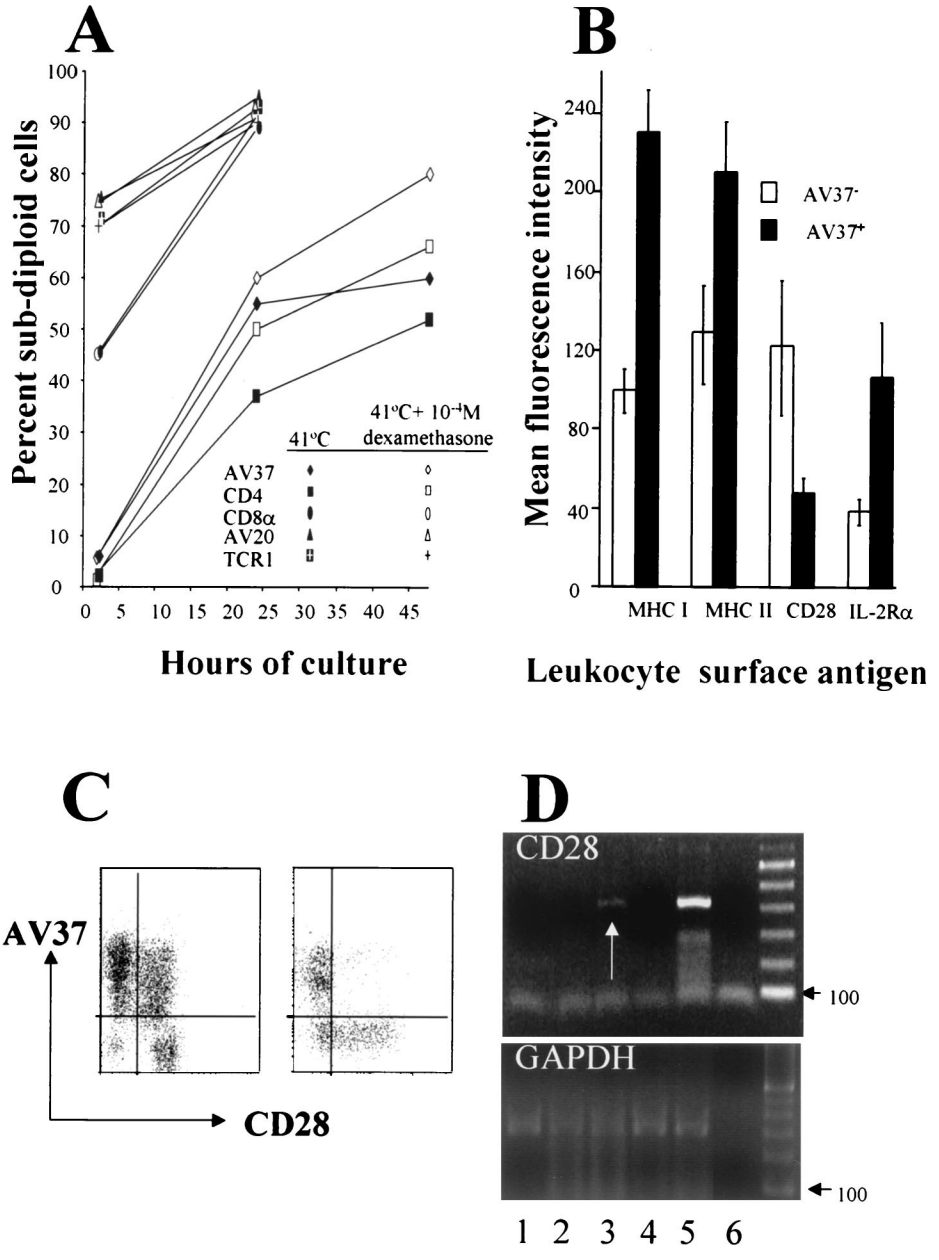

FIG. 6. Protection from cell death in vitro and immune accessory molecule expression by MD lymphoma cell subpopulations. (A) All MD lymphoma cell subpopulations die at a similar rate when cultured in vitro. Proportions of ovarian MD lymphoma cell populations with subdiploid DNA incubated either with or without 10^{-4} M DEX are shown. DEX increased the proportion of all cell types with subdiploid DNA. At 48 h, $AV20^+$, $CD8\alpha^+$, and $TCR1^+$ cells could not be identified in cultures. (B) MHC class I and II, CD28, and IL-2Rα expression levels measured by flow cytometry (means ± standard errors of the means) of any cells recognized by the AV37 MAb ($AV37^+$) and $AV37^-$ lymphoma cell populations. $AV37^+$ MD lymphoma cells expressed more MHC class I and class II and IL-2Rα than did $AV37^-$ cells. $AV37^+$ lymphoma cells had decreased CD28 expression. (C) Flow cytometry dot plots showing decreased (nerve lymphoma) or virtually absent (ovarian lymphoma) CD28 expression by $AV37^+$ MD lymphoma cells. (D) RT-PCR for CD28 and GAPDH mRNA expression. PCR primers were designed for exon sequences of both the chicken CD28 gene (the cDNA template gives a 407-bp product) and the chicken GAPDH gene (the cDNA template gives a 295-bp product). CD28 mRNA was absent from MDCC HP9 (lane 1), MDCC HP18 (lane 2), and MDCC HP89 (lane 4) but present in MDCC IAH8 (lane 3) (white arrow) and ConA-stimulated T cells (lane 5). GAPDH mRNA was present in all samples except negative-control bovine PBL (lane 6). A 100-bp (arrows) DNA ladder is shown to the right of lane 6.

modal in each chicken line; the first (and greatest) peak was at 7 dpi and the second peak was at 28 or 42 dpi, depending on the chicken line. The first peak is during the early cytolytic and immunosuppressive phase (up to 7 dpi); the second peak is at the time of the late immunosuppressive phase (reviewed in reference 77) (and gross lymphoma formation in susceptible chickens). The second immunosuppressive phase may be a cause or a result of the $AV37^{hi}$ leukemia, i.e., immunosuppression may allow proliferation of $AV37^{hi}$ PBL or $AV37^{hi}$ PBL may be immunosuppressive. Regardless, $AV37^{hi}$ PBL are ac-

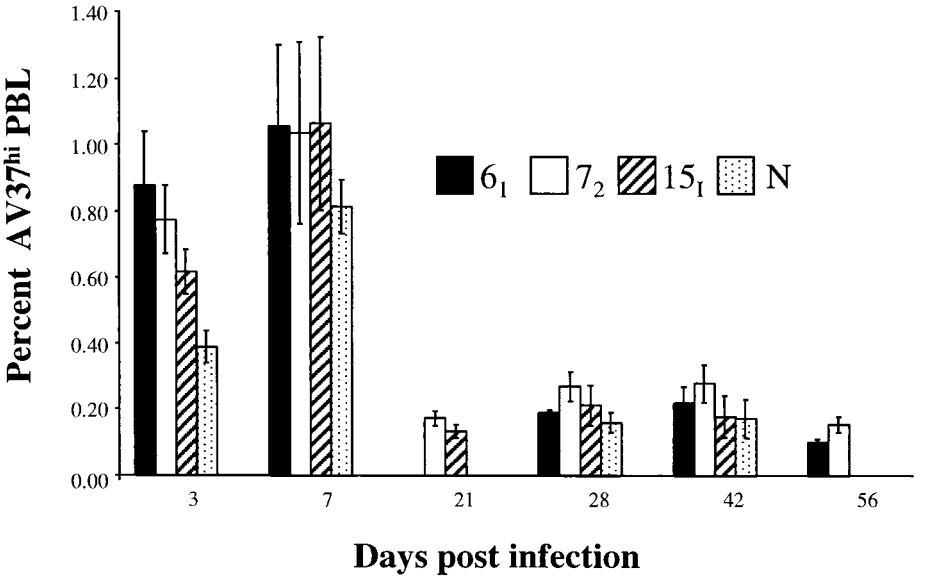

FIG. 7. AV37^{hi} leukocytosis was present in line N and 6_1 (MD resistant) and line 7_2 and 15I (MD susceptible) chickens after MDV infection. Density gradient purified PBL taken at 3, 7, 21, 28, 42, and 56 dpi from 10 infected and 6 uninfected chickens of each genotype were incubated with MAbs AV37 or RSVG-MAb29. Cells (10^6 per sample) were collected by flow cytometry and analyzed (41). The electronic analysis gates were set to analyze each sample for the presence of AV37^{hi} cells (see Materials and Methods). Corrected percentages of AV37^{hi} cells were calculated by subtracting the (negligible) percentages of RSVG-MAb29$^+$ events from the percentages of AV37^{hi} events. AV37^{hi} PBL are not observed in any uninfected chickens of any line.

tivated CD4$^+$ T cells and as such would be expected to migrate into tissues (88). Migration from blood into organs may be the explanation for why AV37^{hi} PBL percentages decreased in all lines between 7 and 21 dpi when the disease entered clinical latency.

TCRVB gene family expression in MD lymphomas suggests multiple, independent, neoplastic transformation events after MDV infection. The chicken has two TCRVB gene families recognized by MAbs TCR2 and TCR3. TCR2$^+$ and TCR3$^+$ cells differ in their development and tissue distribution (reviewed in reference 32) and can respond differently during an immune response to particular pathogens (reviewed in references 83 and 87). It has been suggested that MD lymphomas arise from a single neoplastic transformation event. This is because cells taken from MD lymphomas of multiple organs within the chickens have identical numbers of MDV integration events (notably MD lymphoma cells taken from different chickens have different numbers of MDV integration events) (27). If MD lymphomas were clonal, then TCRVB usage, at least in neoplastically transformed cells, would be clonal.

We examined TCRVB gene family expression from single MD lymphomas (Table 2, birds 1 to 14) and from multiple MD lymphomas in the same chicken (Table 2, birds 15 to 17) by flow cytometry. We found T cells of each TCR family were present in the same MD lymphomas. In all lymphomas examined, proportions of cells from both TCRVB gene families were AV37^{hi}. Generally, one or the other TCRVB gene family was predominant in any given lymphoma, and the AV37^{hi} TCR2$^+$ or TCR3$^+$ subpopulations reflected this predominance. MD lymphoma cells from different lymphomas within the same chicken had different proportions of TCRVB family AV37^{hi} cells (Table 2, birds 15 to 17). In one case (Table 2, bird 17), the proportion of TCR2 AV37^{hi} cells was greater than TCR3 AV37^{hi} cells in one lymphoma but the opposite occurred in another lymphoma of the same chicken.

Our data suggest, in contrast to previous work (27), that MD lymphomas are not clonal but result from multiple neoplastic transformation events. Detailed immunohistochemical analysis of developing lymphoma foci with TCR2 and TCR3 will determine if each developing lymphoma focus is clonal. However, the disadvantage of the MAbs TCR2 and TCR3 is that they can only distinguish between the two TCRVB gene families. An obvious refinement, which will give a better estimation of the degree of neoplastically transformed cell heterogeneity in MD lymphomas, will be to amplify by PCR and then sequence the DNA encoding the TCR hypervariable domains. Because the technology now exists, it is even possible to remove individual AV37^{hi} cells from individual developing MD lymphoma foci and analyze TCR heterogeneity at the single-cell level.

DISCUSSION

Our work provides two key findings that contribute to the fundamental understanding of MD herpesvirus-induced oncogenesis. First, we have definitively identified the neoplastically transformed cells in heterogeneous MD lymphomas in vivo as CD4$^+$, MHC class I^{hi}, MHC class II^{hi}, IL-2Rα^+, CD28$^{lo/-}$, MDV pp38$^-$, gB$^-$, $\alpha\beta$ TCR$^+$ cells. Second, we have demonstrated that overexpression of an antigen, recognized by the MAb AV37, is a unique identifier of these neoplastically transformed cells. This now provides the opportunity to gain a detailed understanding of MD lymphoma pathogenesis in MD-susceptible chickens. It also provides a basis for understanding why lymphomas do not form in MD lymphoma-resistant chick-

ens, even though MD-resistant chickens are as equally vulnerable to lifelong MDV infection as MD-susceptible chickens. Furthermore, our work highlights the utility of MD as an excellent model for understanding herpesvirus oncogenesis in general.

A major factor in abandoning MD as a general model for understanding herpesvirus oncogenesis, and the single biggest impediment to understanding MDV oncogenesis, has been the inability to identify the neoplastically transformed cells within MD lymphomas. This inability to identify the neoplastically transformed cells in MD lymphomas occurred despite many attempts having been made to do so since the advent of MAb. Much effort was invested in the phenotypic description of MDCC derived from either MD lymphomas or induced local lesions (reviewed in reference 65). The major drawback of this approach is that MDCC do not necessarily represent those neoplastically transformed cells in MD lymphomas in vivo. MDCC are MDV-infected cells that have acquired adaptive (but as yet undefined) mutations and require specific culture conditions to allow their transition to survival in an in vitro environment. MDCC are useful as a starting point, but none of the many phenotypes defined by using MDCC can be used to definitively identify the neoplastically transformed cells in MD lymphomas in vivo. We took an alternative approach and identified the neoplastically transformed MD lymphoma cells directly ex vivo.

Our identification of the neoplastically transformed MD lymphoma cells strictly required that such cells absolutely conform to four generally accepted and essential criteria for neoplastic transformation in vivo. The criteria are (i) hyperproliferation and yet (ii) protection from the default pathway of AICD in vivo (31), (iii) latent MDV infection (because herpesvirus replication in productive infections results in obligate cytolysis [17, 82]), and (iv) ubiquity in MD lymphomas. Using only the MAb AV37, we were able to identify cells which fulfill the essential criteria for neoplastic transformation in vivo. Specifically, such cells grossly overexpress the antigen recognized by the MAb AV37 (i.e., they are $AV37^{hi}$). $AV37^{hi}$ MD lymphoma cells have extremely high proliferation rates yet extremely low levels of death. Furthermore, the high-level MDV infection of $AV37^{hi}$ MD lymphoma cells (15, 85) is a latent infection. Finally, and in common with findings from other work (14, 15, 85), $AV37^{hi}$ cells are frequent cells in all MD lymphomas.

Three other findings with the identical model system support our assertion that AV37 antigen overexpression identifies the neoplastically transformed cells in MD lymphomas. First, $AV37^{+}$ cells in MD lymphomas (equivalent to $AV37^{hi}$ cells in our present work) express the highest levels of mRNA for *meq*, the putative MDV oncogene (85). Second, when analyzed for expression of *meq* protein with MAb 23b46 (85) (a gift from L. F. Lee), the same MDCC that we used in the present work are $AV37^{hi}$ and $23b46^{hi}$ (unpublished data). Third, $AV37^{hi}$ cells taken from MD lymphomas directly ex vivo are also $23b46^{hi}$ (S. C. Burgess, L. J. N. Ross, and T. F. Davison, 4 November 1999, European Patent Office, WO99/55860; S. C. Burgess, J. R. Young, and T. F. Davison, Proc. 6th Avian Immunol. Res. Group Meet., abstr. 232, 2000; unpublished data).

Although minute proportions of leukocytes isolated from uninfected chickens may be $AV37^{+}$, the relative expression level of the antigen by such cells is at least 100-fold less than that of $AV37^{hi}$ MD lymphoma cells, i.e., uninfected leukocytes are $AV37^{lo}$. Furthermore, leukocytes remain $AV37^{lo}$ after mitogen activation. Crucially B, $CD8\alpha^{+}$, or $TCR1^{+}$ MD lymphoma cells, which have high levels of MDV antigen expression and death, are $AV37^{lo}$. Because the AV37 antigen is host encoded and overexpressed after MDV infection only by neoplastically transformed cells, it must belong to one of three defined tumor antigen families (9, 10, 28, 72, 73, 103). The AV37 antigen must be either a tumor-specific shared antigen, an overexpressed normal antigen, or a differentiation antigen. (Unpublished data suggest that the AV37 antigen is a member of the tumor necrosis factor receptor family.) Other host-encoded molecules may also be overexpressed by neoplastically transformed MD lymphoma cells, and any of the molecules known collectively as MATSA (37, 43, 52, 55, 59, 63, 64, 68, 84, 91, 98, 100, 108) may be examples.

One implication of low-level AV37 antigen expression by uninfected leukocytes is that AV37 antigen overexpression may occur after neoplastic transformation by other oncogenic leukotropic chicken viruses (unpublished observations). AV37 antigen up-regulation may even be an evolutionarily conserved response by host cells to neoplastic transformation by lymphotrophic viruses in many species (unpublished data). However, we never found $AV37^{+}$ uninfected myeloid cells, and we predict that the neoplastically transformed cells within myeloid tumors would not overexpress the AV37 antigen.

In addition, we have made several observations relevant to potential immunity against developing MD lymphomas: our description of myeloid cells surrounding and within developing MD lymphomas and our observations regarding MHC class I and class II as well as CD28 antigen expression by MD lymphoma cells.

Myeloid cells surround and are present within developing MD lymphomas and may have at least two potential roles in controlling MD lymphoma. First, as cells of the innate immune system, they may be present as part of an inflammatory response and may directly kill neoplastically transformed cells. Second, they may present antigens bound to MHC class I and class II to cells of the acquired immune system. This antigen-presenting function may be local or may occur after transit from the lymphoma environment to lymphoid organs. Regardless, this antigen-presenting potential suggests a mechanism to promote primary acquired immunity against cells expressing immunogens within MD lymphoma. Any such acquired immunity may be central to restricting lymphoma development in MD-resistant chickens, whereas evasion of such acquired immunity could allow MD lymphoma development in susceptible chickens.

Evidence of immune evasion mechanisms was our fifth desirable criterion for the neoplastically transformed cells in MD lymphomas. Immune evasion mechanisms are advantageous for any neoplastically transformed cell but are essential for neoplastically transformed cells expressing foreign antigens. $AV37^{hi}$ MD lymphoma cells contain high latent MDV loads (15, 85) and, because latently MDV-infected cells express latent MDV antigens (62), so must $AV37^{hi}$ MD lymphoma cells. However, we found that $AV37^{hi}$ MD lymphoma cells do not down-regulate MHC class I and so cannot use this immune

evasion mechanism as productively MDV-infected cells are suggested to do (42). Indeed, the AV37^{hi} MD lymphoma cells express higher levels of MHC class I and class II than do AV37$^{-/lo}$ MD lymphoma cells. Unless actively prevented from doing so, AV37^{hi} cells should present peptides derived from MDV antigens (and/or abnormally expressed host-encoded antigens) bound to MHC class I. This means that AV37^{hi} MD lymphoma cells are excellent potential cytotoxic T lymphocyte targets. An obvious and essential question to answer, therefore, is how, in the presence of myeloid cells and with the high potential to express antigens, do AV37^{hi} MD lymphoma cells survive in MD-susceptible chickens?

AV37^{hi} MD lymphoma cells must employ immune evasion mechanisms, and we have direct evidence that AV37^{hi} MD lymphoma cells down-regulate CD28 at the mRNA level. This CD28 down-regulation may be directly mediated by MDV. Regardless, CD28 down-regulation is extremely notable because CD28 is an essential costimulatory molecule for T-cell activation (33, 90), and CD28 down-regulation is a recognized pathogen immune evasion mechanism (reviewed in reference 94). AV37^{hi} MD lymphoma cells have an excellent potential to present antigens. The CD28 down-regulation by AV37^{hi} cells could directly remove costimulation to T cells (11), induce tolerance to AV37^{hi} MD lymphoma cells in vivo (2), and/or cause an inappropriate cytokine environment within MD lymphoma (81).

Furthermore, MD lymphoma structure (i.e., AV37^{hi} cell clusters surrounded by [AV37$^{-/lo}$] CD4^{+} cells) (14) together with the results in this paper suggest that the greatest potential TCR-MHC class II interactions are between AV37^{hi} CD4^{+} cells, followed by those between AV37^{hi} and AV37$^{-/lo}$ CD4^{+} cells. We speculate, based on evidence from other systems, that such interactions could favor T helper (Th2) cytokine production (56), induce death and/or anergy in lymphocytes attracted to developing MD lymphomas (36), or promote regulatory T-cell development (99). All of these suggested mechanisms are antagonistic to cell-mediated immunity. Each proposed immune evasion mechanism, and others that we have not suggested, should be examined in detail. Our work provides a basis for doing so.

We have identified the neoplastically transformed cells in MD lymphomas and described one potential immune evasion mechanism for these cells. However, we also demonstrate for the first time that, based on TCRVB gene expression, multiple neoplastic transformation events must occur after MDV infection, that the local lymphoma environment is essential for cell survival in MD lymphomas, and that neoplastically transformed PBL develop soon after MDV infection in both MD-resistant and -susceptible chickens. Each of these findings is important for further understanding MD lymphoma pathogenesis

More specifically, our work, together with the MDV genome sequences (45, 53, 102) and the chicken leukocyte expressed sequence tag database (101), provides a foundation for a detailed investigation of both host-virus and cell-cell interactions in MD lymphoma. There are two obvious priorities. First, host gene transcription in latently infected cells that are neoplastically transformed should be compared with that in latently infected cells that are not neoplastically transformed and with that in (non-MDV infected) activated T helper cells. Second, the MDV latency program(s) in latently infected cells that are neoplastically transformed should be compared with that in latently infected cells that are not neoplastically transformed. In this context, our work emphasizes first that the highest levels of *meq* (the strongest candidate MDV oncogene) (50) mRNA, demonstrated to be in AV37^{+} MD lymphoma cells by in situ hybridization (85) are in the neoplastically transformed MD lymphoma cells; second, we demonstrate that MDV pp38 cannot be involved in neoplastic transformation in vivo.

Our work is obviously applicable to poultry production, where, despite intensive vaccination, MDV cyclically reemerges as a significant problem (106, 107). However, it also has a broader impact. To date, the lack of amenable natural animal models has impeded the general understanding of herpesvirus lymphoma pathogenesis. For the first time, it now possible to examine the mechanisms involved in herpesvirus tumorigenesis and/or tumor immunity in a natural animal model either in vivo or directly ex vivo . MD, the first naturally occurring neoplastic disease to be controlled by antiviral vaccination, was used as an animal model in the 1970s and early 1980s. However, the MD model fell out of favor because molecular mechanisms of virus-host intracellular pathogenesis were recognized to differ between MDV and Epstein-Barr virus and because of the inability to identify the neoplastically transformed cells within MD lymphomas. Moreover, the advent of genetically engineered mice allowed cultured human lymphoma cells to survive after transplant. A disadvantage of mouse models is that the lymphoma cells are xenografts of in vitro-cultured cells. Natural mechanisms of herpesvirus oncogenesis and gross tumorigenesis (or not) can only be investigated in the context of an immune system which has coevolved with the pathogen. Our work reestablishes the utility of MD as a natural model of susceptibility and resistance to herpesvirus-induced tumorigenesis.

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